USM SHORT-TERM PROJECT FINAL REPORT

FEBRUARY 2005

PROJECT TITLE:

ACUTE EFFECTS OF AMMONIA ON 'CITRULLINE - NO CYCLE ENZYMES', ARGINASE AND RELATED METABOLITES IN DIFFERENT REGIONS OF RAT BRAIN

NAME OF INVESTIGATOR:

Dr. Mummedy Swamy

CO-INVESTIGATOR:

En. Chandran Govindasamy

Department of Chemical pathology,

School of Medical Sciences,

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Laporan Akhir Projek Peyelidikan [USM JP – 06]

Laporan Akhir Projek Peyelidikan [USM JP – 06]

BAHAGIAN PENYELIDIKAN & PEMBANGUNAN
CANSELORI
UNIVERSITI SAINS MALAYSIA

Laporan Akhir Projek Penyelidikan Jangka Pendek

1) Nama Penyelidik: **DR. MUMMEDY SWAMY**

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Nama Penyelidik-Penyelidik Lain (Jika berkaitan) : En. CHANDRAN GOVINDASAMY

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2) Pusat Pengajian/Pusat/Unit : Department of Chemical pathology,

School of Medical Sciences, Health Campus, USM

3) Tajuk Projek:

ACUTE EFFECTS OF AMMONIA ON "CITRULLINE - NO CYCLE ENZYMES ", ARGINASE AND RELATED METABOLITES IN DIFFERENT REGIONS OF RAT BRAIN.

4) (a) Penemuan Projek/Abstrak

(Perlu disediakan makluman di antara 100 – 200 perkataan di dalam Bahasa Malaysia dan Bahasa Inggeris. Ini kemudiannya akan dimuatkan ke dalam Laporan Tahunan Bahagian Penyelidikan & Pembangunan sebagai satu cara untuk menyampaikan dapatan projek tuan/puan kepada pihak Universiti).

ACUTE EFFECTS OF AMMONIA ON 'CITRULLINE-NO CYCLE ENZYMES', ARGINASE AND RELATED METABOLITES IN DIFFERENT REGIONS OF RAT BRAIN

ABSTRACT

Nitric oxide (NO) is involved in many physiological and pathological processes in the brain. NO is synthesized from arginine by nitric oxide synthase (NOS) enzymes. Citrulline, which is formed as a by-product of the NOS reaction, can be recycled to arginine by successive actions of argininosuccinate synthetase (ASS) and argininosuccinate lyase (ASL) via the citrulline-NO cycle. Hyperammonemia is known to cause poorly understood perturbations of the citrulline-NO cycle. Both ASS and ASL genes are reported to be induced in astrocytes but not in neurons of aggregates exposed to 5 mM ammonium chloride, suggesting that hyperammonemic brain might increase its recycling of citrulline to arginine. To understand the role of citrulline-NO cycle in hyperammonemia, NOS, ASS, ASL and arginase activities, as well as nitrate/nitrite (NOx), the stable end products of NO, and other related metabolites were estimated in cerebral cortex (CC), cerebellum (CB) and brain stem (BS) of rats subjected to acute ammonia toxicity (0.8mmol of ammonium acetate per 100g body weight). NOx concentration and NOS activity were found to increase in all the regions of brain in acute ammonia toxicity. The activities of ASS (CC, CB and BS) and ASL (CC and CB) also showed an increase whereas the activity of arginase was not changed. The concentrations of arginine and ornithine were increased in all the regions of brain in acute ammonia

toxicity whereas citrulline concentration was not changed. Glutamine concentration was significantly increased in all regions of brain while glutamate and GABA concentrations were not changed. The results of this study clearly demonstrated the increased formation of NO, suggesting the involvement of NO in the pathophysiology of acute ammonia toxicity. The increased activities of ASS and ASL enzymes indicate the increased and effective recycling of citrulline to arginine in acute ammonia toxicity, making NO production more effective and contributing to its toxic effects.

AKUT KESAN AMMONIA TERHADAP 'ENZIM-ENZIM DALAM KITARAN CITRULLINE-NO', ARGINASE DAN LAIN-LAIN METABOLIT DALAM PELBAGAI BAHAGIAN OTAK TIKUS

ABSTRAK

Nitrik oksida (NO) terlibat dalam pelbagai proses fisiologi dan patologi di dalam otak. NO disintesiskan daripada arginine oleh enzim-enzim nitric oxide synthase (NOS). Citrulline yang terbentuk sebagai hasil sampingan daripada tindakbalas NOS, boleh dikitar semula kepada arginine oleh tindakbalas-tindakbalas argininosuccinate synthetase (ASS) dan argininosuccinate lyase (ASL) melalui kitaran citrulline-NO. Walau bagaimanapun, kesan keadaan ammonia yang tinggi terhadap kitaran citrulline-NO ini tidak dapat difahami dengan tepat. Dalam kajian yang lepas, kedua-dua gen ASS dan ASL didapati teruja dalam astrocytes, tetapi tidak dalam neurons, yang didedahkan kepada ammonium chloride pada kepekatan 5mM. Ini menunjukkan kandungan ammonia yang tinggi di dalam otak menyebabkan peningkatan pembentukan citrulline kepada arginine. Untuk memahami peranan kitaran citrulline-NO dalam keadaan ammonia yang tinggi, aktiviti-aktiviti NOS, ASS, ASL dan arginase serta kepekatan nitrat/nitrit (NOx) dan lain-lain metabolit dianggarkan di dalam korteks serebral, serebellum dan batang otak tikus yang telah dikenakan ketoksikan ammonia akut (0.8mmol ammonium acetate bagi setiap 100g berat badan). Kepekatan NOx dan aktiviti NOS didapati meningkat di dalam semua bahagian otak tikus dalam keadaan ketoksikan ammonia akut. Aktiviti-aktiviti ASS dan ASL juga menunjukkan peningkatan yang setara tetapi aktiviti arginase tidak berubah. Kepekatan arginine dan ornithine dalam pelbagai bahagian otak yang

mengalami ketoksikan ammonia akut menunjukkkan peningkatan tetapi tidak pada kepekatan citrulline. Kepekatan glutamine menunjukkan peningkatan yang ketara dalam semua bahagian otak tetapi kepekatan glutamate dan GABA tidak menunjukkan perubahan yang ketara. Keputusan yang didapati dari eksperimen ini jelas menunjukkan penghasilan NO yang tinggi ini menyumbangkan kepada penglibatan NO dalam patofisiologi ketoksikan ammonia akut. Peningkatan dalam aktiviti-aktiviti ASS dan ASL menunjukkan peningkatan dan keberkesanan kitaran semula citrulline kepada arginine dalam ketoksikan ammonia akut, juga menyebabkan penghasilan NO menjadi lebih berkesan dan seterusnya menyumbangkan kesan toksiknya.

(b) Senaraikan Kata Kunci yang digunakan di dalam abstrak:

Bahasa Malaysia	Bahasa Inggeris
Otak tikus	Rat brain
Ketoksikan ammonia akut	Acute ammonia toxicity
Nitrik oxida	Nitric oxide
Kitaran citrulline-NO	Citrulline-NO cycle
Arginase	Arginase
Nitrat/nitrit (NOx)	Nitrate/Nitrite (Nox)

5) Output Dan Faedah Projek

(a) Penerbitan (termasuk laporan/kertas seminar)
(Sila nyatakan jenis, tajuk, pengarang, tahun terbitan dan di mana telah diterbit/dibentangkan).

1) Adlin Zafrulan Zakaria, Chandran Govindasamy, H. A. Nadiger and Mummedy Swamy (2004) Effects of acute ammonia toxicity on Nitric oxide and "Citrulline-NO cycle enzymes" in rat brain. 29th Annual conference of the Malaysian Society for Biochemistry and Molecular Biology (MSBMB). 28th & 29th Sept 2004, Nikko Hotel, Kuala Lumpur

2) Adlin Zafrulan Zakaria (2004) Effects of acute ammonia toxicity on Nitric oxide (NO), Citrulline-NO cycle enzymes, arginase and related metabolites in different regions of rat brain. A Dissertation submitted in partial fulfillment of the requirements for the Degree of Master of Pathology (Chemical Pathology) Universiti Sains Malaysia, November 2004.

3) M. Swamy, Adlin Zafrulan Zakaria, Chandran Govindasamy and H. A. Nadiger (2005) Effects of acute ammonia toxicity on Nitric oxide (NO), Citrulline-NO cycle enzymes, arginase and related metabolites in different regions of rat brain. Paper submitted for publication in Molecular Genetics and Metabolism.

(b) Faedah-Faedah Lain Seperti Perkembangan Produk, Prospek Komersialisasi Dan Pendaftaran Paten.
(Jika ada dan jika perlu, sila guna kertas berasingan)

Nil

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(c) Latihan Gunatenaga Manusia

i) Pelajar Siswazah: Dr. Adlin Zafrulan Zakaria- A Student of M. Path

Chemical pathology trained to complete his dissertation

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ii) Pelajar Prasiswazah: Nil.

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iii) Lain-Lain : Nil

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6. Peralatan Yang Telah Dibeli:

Nil

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UNTUK KEGUNAAN JAWATANKUASA PENYELIDIKAN UNIVERSITI

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T/TANGAN PENGERUSI
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Laporan Komprehensif

Laporan Komprehensif

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Title:

ACUTE EFFECTS OF AMMONIA ON 'CITRULLINE - NO CYCLE ENZYMES', ARGINASE AND RELATED METABOLITES IN DIFFERENT REGIONS OF RAT BRAIN

Name of Investigator: **Dr. Mummedy Swamy**

Co-investigator: En. Chandran Govindasamy

Student: Dr. Adlin Zafrulan Zakaria

Department of Chemical pathology,

School of Medical Sciences,

USM

Introduction:

Nitric oxide (NO) is involved in many physiological and pathological processes in the brain. NO is synthesized from arginine by nitric oxide synthase (NOS), and the citrulline generated as a by-product can be recycled to arginine by argininosuccinate synthetase (AS) and argininosuccinate lyase (AL) via the citrulline-NO cycle (Zhang et al 2000). Generation of nitric oxide, NO, a versatile molecule in signaling processes and unspecific immune defense, is intertwined with synthesis, catabolism and transport of arginine which thus ultimately participates in the regulation of a fine-tuned balance between normal and

pathophysiological consequences of NO production (Wiesinger 2001). Co-induction of inducible nitric oxide synthase and arginine recycling enzymes in cytokine-stimulated PC12 cells and high output production of nitric oxide is reported by Zhang et al (2000). Co-induction of argininosuccinate synthetase, cationic amino acid transporter-2, and nitric oxide synthase in activated murine microglial cells was reported by Kavahara etal (2001). The mechanism of action of ammonia toxicity in brain is not clearly understood (Sadasivudu et al 1983). However, alterations in metabolic and functional aspects of brain such as depression in energy metabolism , changes in neurotransmitter levels,involvement of glial cells,changes in the activity of Na+ -K+-activated ATPase affecting the membrane function(Sadasivudu et al 1977), and alterations in blood-brain barrier(Horowittz 1981) have been implicated. Hyperammonemia is known to cause poorly understood perturbations of the citrulline-NO cycle. It is reported by Braissant etal (1999) that both AS and AL genes are induced in astrocytes but not in neurons of aggregates exposed to 5 mM NH4Cl, suggesting the hyperammonemic brain might increase its recycling of citrulline to arginine. In order to understand the role of citrulline-No cycle in ammonia toxicity the proposed study was undertaken.

Objectives:

General objectives:

The objective of the study was to understand the role of citrulline-nitric oxide cycle in three different regions of rat brain; cerebral cortex, cerebellum and brain stem, subjected to acute ammonia toxicity.

Specific objectives:

1. To determine the concentration of ammonia in different regions of rat brain subjected to ammonia toxicity.
2. To determine the changes in the concentration of nitric oxide in different regions of rat brain subjected to acute ammonia toxicity.
3. To determine the changes in the activities of the citrulline-NO cycle enzymes; NOS, ASS, ASL, and activity of arginase in different regions of rat brain subjected to acute ammonia toxicity.
4. To determine the changes in the concentrations of arginine, citrulline, ornithine, glutamate, glutamine and gamma-amino butyric acid (GABA) in different regions of rat brain subjected to acute ammonia toxicity.

Materials and Methods:

Experimental design:

Sprague Dawley rats were used as subjects. Male rats weighing 200 – 350 grams were used for the study and divided into two groups; the control group and the test group (acute ammonia toxicity group). Ammonia toxicity was produced by the procedure of Bessman & Paul (1976) as described by Swamy & Sadasivudu (1983). This was done by administrating ammonium acetate at a dose of 0.8mmol/100grams body weight intraperitoneally. Acute toxic effects of ammonia were then studied in these animals after 30 minutes of that injection. The control animals were given normal saline instead of ammonium acetate and also sacrificed 30 minutes after the intraperitoneal injection.

The rats were sacrificed using the guillotine and the brains were quickly removed according to the procedure described by Sadasivudu & Lajtha (1970). This was done by making an incision in the middle of the skull followed by two more incisions laterally on both sides. Then the skull was flipped open and the brain was scooped out. After that the three different regions of the brain; cerebral cortex, cerebellum and brain stem were separated and put in normal saline to clear off the blood clots. Each brain region was then wrapped in the aluminium foil, labelled and kept in ice box before homogenization.

Each of the brain regions was weighed and used for preparation of homogenates in 0.05M phosphate buffer. Homogenate preparation was done using a tefflon pestle. Ten percent (10%) weight/volume homogenates were prepared for ammonia, NO, NOS, arginase and amino acids analysis while 20% homogenates were prepared for estimation of ASS and ASL activities. All the parameters were assessed using 6 animals independently from each group.

Estimation of ammonia:

Ammonia concentration in brain tissue homogenates was estimated using the enzymatic assay with glutamate dehydrogenase, using the ammonia kit from Randox. Ammonia combines with α-ketoglutarate (α-KG) and NADPH in the presence of glutamate dehydrogenase (GLDH) to yield glutamate and $NADP^+$. The corresponding decrease in the absorbance (A) of $NADP^+$ at 340nm is proportional to the ammonia concentration.

$$\alpha\text{-KG} + NH_3 + NADPH \xrightarrow{GLDH} \text{Glutamate} + NADP^+$$

The concentration of ammonia is calculated using the formula below,

$$\frac{A_{sample} - A_{blank}}{A_{standard} - A_{blank}} \times 294$$

and this would give the concentration of ammonia in umol/l. The ammonia concentrations were however expressed as umol/g wet weight of tissue.

For the estimation of ammonia, 10% (weight/volume) tissue homogenates were used i.e. using 10grams of brain tissue in 100mls of phosphate buffer. Therefore, in a liter (1000mls) of buffer, there should be 100grams of brain tissue. From this we can conclude that umol/l is equal to umol/100grams tissue:

$$\frac{\text{umol/l}}{100} = \text{umol/g wet weight of tissue}$$

Estimation of Nitric oxide (NO)

NO was estimated as nitrate/nitrite (NOx) by Griess reaction after conversion of nitrate to nitrite by nitrate reductase, using the Nitric Oxide Assay Kit from Calbiochem (Catalogue number 482650). This kit used a colorimetric method in which nitrite was measured to determine the concentration of nitric oxide. In aqueous solution, NO is rapidly converted to nitrate and nitrite. The ratio of nitrate and nitrite concentrations produced from NO may vary substantially depending on the biological fluids. Hence, for accurate assay of total NO generated, both nitrate and nitrite levels must be monitored.

The Griess reaction is based on the chemical reaction, which uses sulfanilamide (Reagent 1) and N-1-napthylethylenediamine dihydrochloride (NED) (Reagent 2) under acidic (phosphoric acid) conditions (Figure 2). The azo compound formed can be measured spectrophotometrically at 540nm. This system detects NO_2^- in a variety of biological and experimental liquid matrices such as plasma, serum, urine, tissue homogenate and tissue culture medium. Spectrophotometric quantitation of nitrite using the Griess reagent was a straightforward method; however, it did not measure nitrate. Therefore, the NADH-dependent enzyme nitrate reductase (NR) was used to convert the nitrate to nitrite prior to quantitation using the Griess reagent.

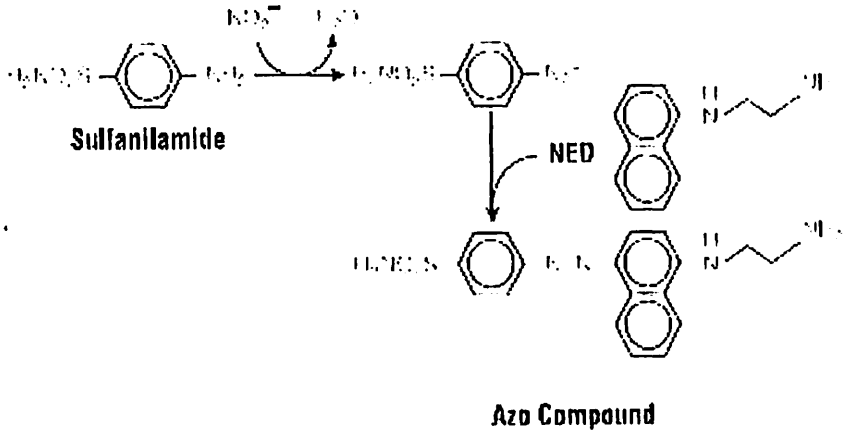

Figure 2: Chemical Reactions Involved in the Measurement of Nitrite (NO_2^-) Using the Griess Reagents (Source: Internet [http: //www.promega.com/tbs/tb229/tb229.pdf]).

The assay was performed in a microtiter plate together with nitrate standard. 40ul of the 10% tissue homogenate and 45ul of buffer (given in the kit) was added so that the final volume is 85ul. Then, 5ul of reconstituted nitrate reductase was added to each well, followed by 10ul of 2mM NADH, before it was incubated for 20 minutes at room temperature in the dark. For the colour development, 20ul each of Griess reagent 1 and 2 were added and after 5 minutes, the absorbance was read at 540nm using the microtiter plate reader. The concentration of nitrite was then obtained from the standard curve (Figure 3.1) by comparing the value of the absorbance. Finally, the concentration of NOx was expressed in nmol/g wet tissue.

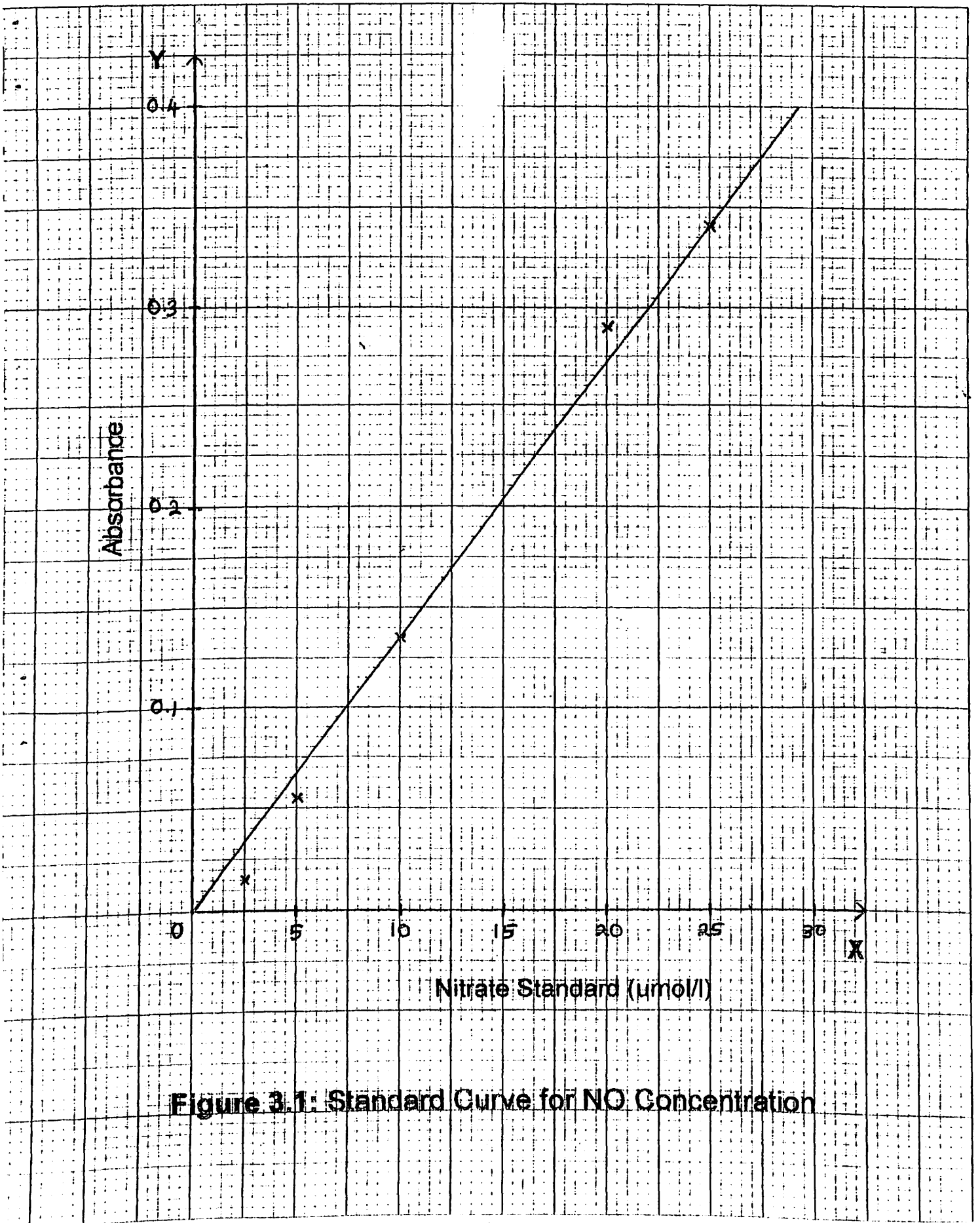

Figure 3.1: Standard Curve for NO Concentration

Enzyme assays:

Nitrc oxide synthase (NOS) activity:

NOS activity was estimated by the method of Yui *et al* (1991) in which the stable end products, NOx, were estimated using the Nitric Oxide Synthase Assay Kit from Calbiochem (Catalogue Number 482702). This kit used colorimetric method in which nitrite concentration was measured to determine the NOS activity. In biological system, NOS acts on L-arginine to produce nitric oxide and citrulline. In this reaction NADPH acts as an essential co-factor. Unfortunately, NADPH can interfere with Griess reagents that are commonly used for nitrite detection, and lead to lower colour yield. In this kit, the limitation was overcome by using lactate dehydrogenase (LDH) to remove excess NADPH. A heat-inactivated control was included for each NOS preparation to serve as a control to measure endogenous nitrate and nitrite. In order to quantitate the nitrite concentration, a nitrate standard curve was performed. The assay was performed in a microtiter plate together with nitrate standard and after the colour developed, absorbance was read at 540nm using the microtiter plate reader.

In the assay, 40ul of the 10% tissue homogenate was used and 20ul of distilled water was added so that the final volume was 60ul. Then 10ul of freshly prepared NADPH solution (1mM) was added to each well, followed by 10ul of nitrate reductase, and the mixture was incubated for 1 hour at room temperature. After that 10ul cofactors and 10ul LDH were added to each well, and the incubation was continued for another 20 minutes. For the colour development, 50ul of Griess reagent 1 and 50ul of Griess reagent 2 were added into each well. After 10 minutes, the absorbance was read and the value of absorbance was then compared with the standard curve (Figure 3.2) to determine the nitrite concentration.

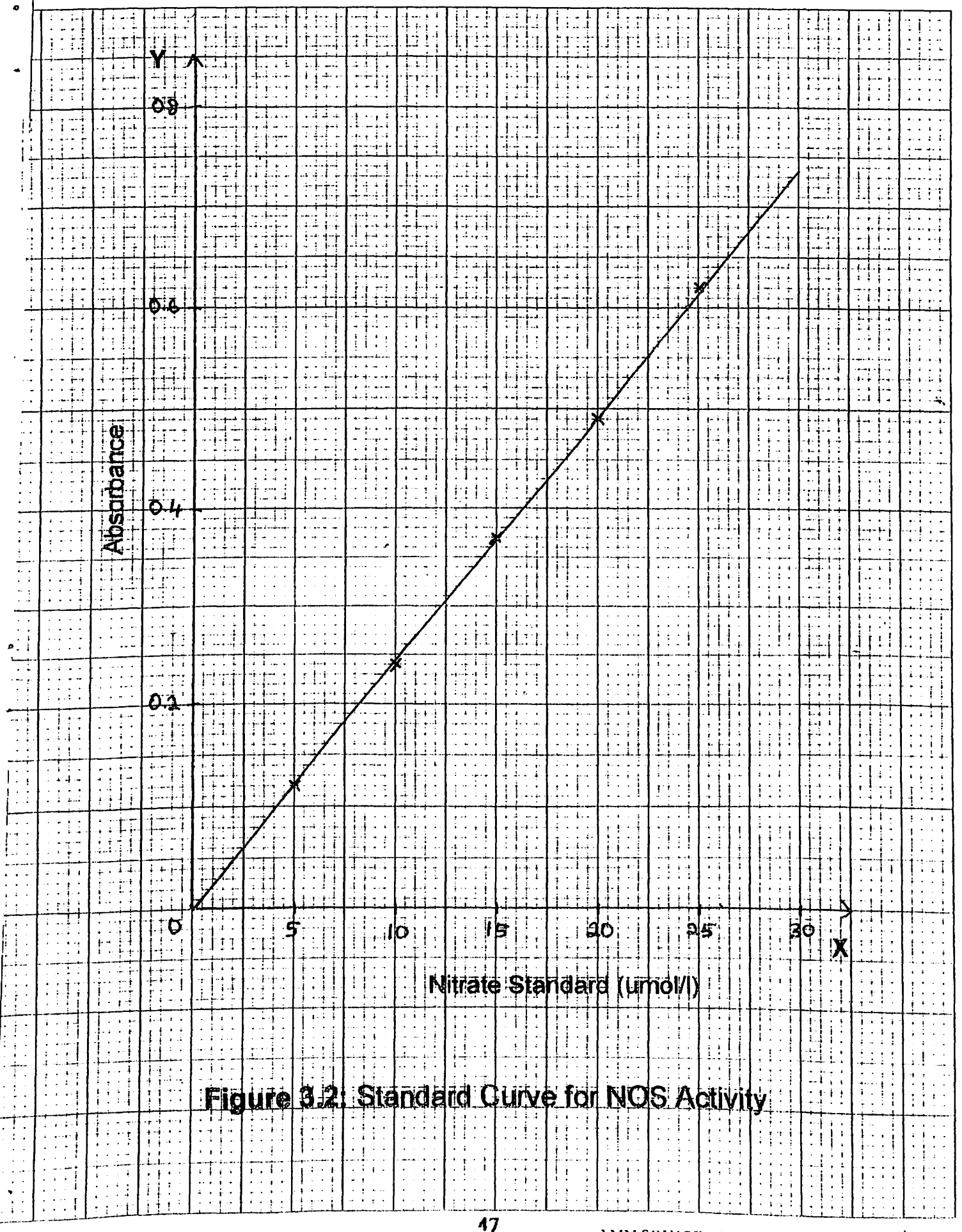

Figure 3.2: Standard Curve for NOS Activity

Finally, the enzyme activity was expressed in nmol of NOx formed per gram wet weight of tissue per hour (nmol NOx/g wet tissue/hr).

Argininosuccinate synthetase (ASS) activity:

Argininosuccinate synthetase activity was estimated by the modified method of Levin (1971) as described by Sadasivudu and Indira Rao (1974). In the estimation of ASS, the incubation mixture contained 0.8ml of 0.01M each of citrulline, aspartic acid, ATP, magnesium chloride, and 21U of arginase, in 0.05M phosphate buffer (pH 7.3). Reaction was started with the addition of 0.2ml of 20% homogenate and incubated at 37^0C for 1 hour. At the end of incubation period, 0.2ml of 50% trichloroacetic acid was added to stop the reaction. For controls, trichloroacetic acid was added before the incubation. The mixture was then centrifuged and 0.5ml of supernatant was used for colour development. The supernatant was mixed with 1.5ml of acid mixture (one part of concentrated sulphuric acid and three parts of concentrated phosphoric acid) and 0.1ml of isonitrosopropiophenone (5% in absolute alcohol). It was kept in boiling water bath for 30 minutes, and then after cooling the tubes, absorbance was read at 540nm. Simultaneously, a urea standard was set up by adding to the standard 0.5ml of substrate mixture and 0.5ml of water. The colour that developed was read at 540nm against the reagent blank. The net absorbance of the sample was then compared with the standard urea curve (Figure 3.3) and the amount of urea was determined. The enzyme activity was expressed as μmol of urea formed per gram wet weight of tissue per hour (μmol urea/g wet tissue/hr).

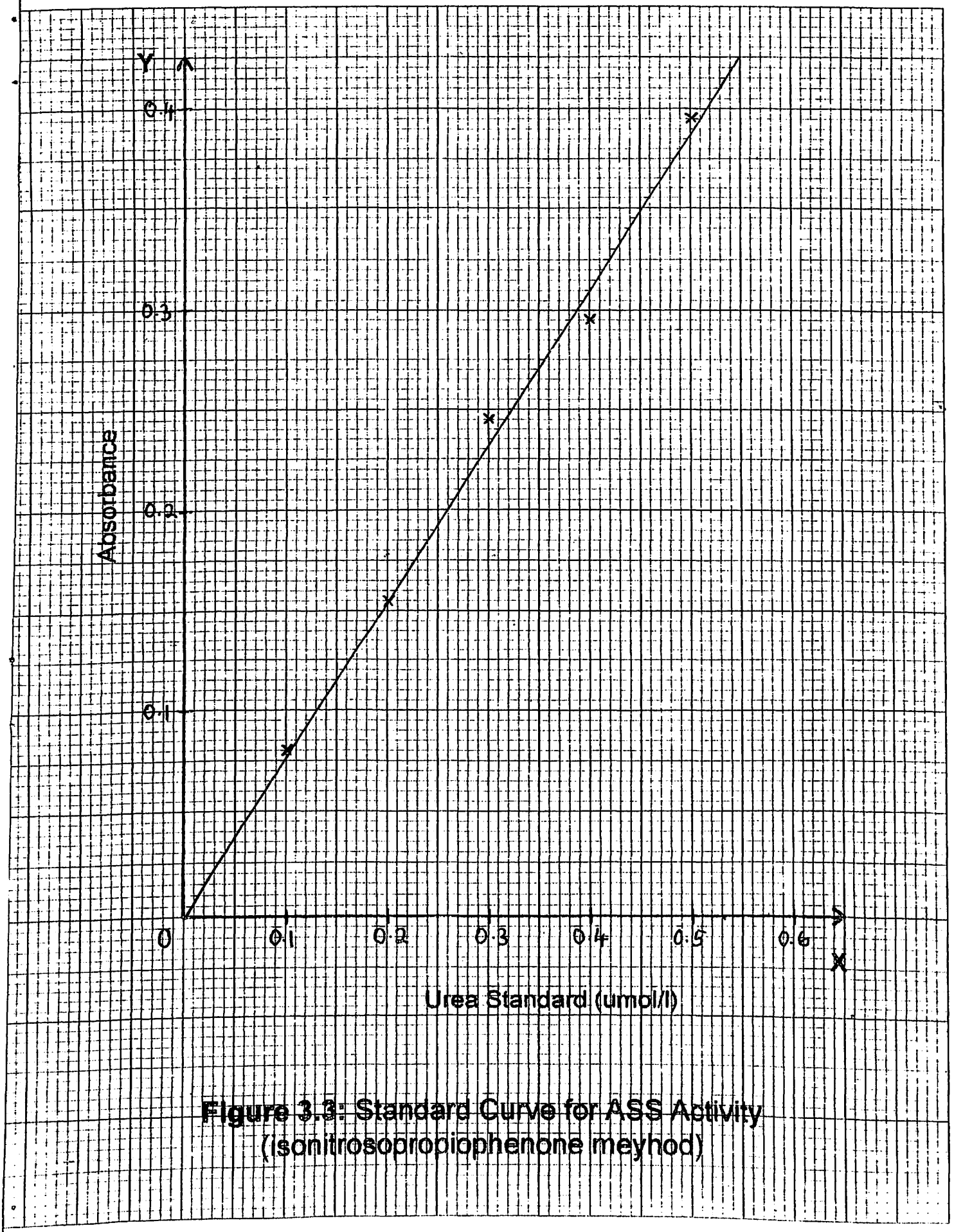

Figure 3.3: Standard Curve for ASS Activity (Isonitrosopropiophenone meyhod)

2 MM SQUARE - 20 CM X 24 CM 40 S

Argininosuccinate layase (ASL) activity

Argininosuccinate layase activity was also assayed by the method of Levin (1971) as described by Sadasivudu and Indira Rao (1974). The assay system for ASL consisted of 0.3ml of 1M phosphate buffer (pH 7.3), 0.6ml of argininosuccinate (6.0mM), 0.2ml of 20% homogenate, and 0.1 ml of arginase (10.5U). At the end of 1 hour incubation at 37^0C, the reaction was stopped by the addition of 0.3ml of 50% trichloroacetic acid. Controls were identical, except that trichloroacetic acid was added before incubation. After incubation, the mixture was centrifuged at 3000rpm for 5 minutes and 0.5ml of the supernatant was used for urea estimation.

Urea was estimated by the modified diacetylmonoxime (DAM) method. In this method, 0.5ml of the supernatant was added with 1.0ml of acid reagent and 1.0ml of DAM reagent, and kept boiling for 10 minutes in water bath. At the end of 10 minutes, the test tubes were cooled under running tap water and the absorbance was read against blank reagent at 540nm wavelength. By using the standard urea curve (Figure 3.4), the amount of urea can be estimated by comparing the absorbance value. The enzyme activity was then expressed as μmol of urea formed per gram wet weight of tissue per hour (μmol urea/g wet tissue/hr).

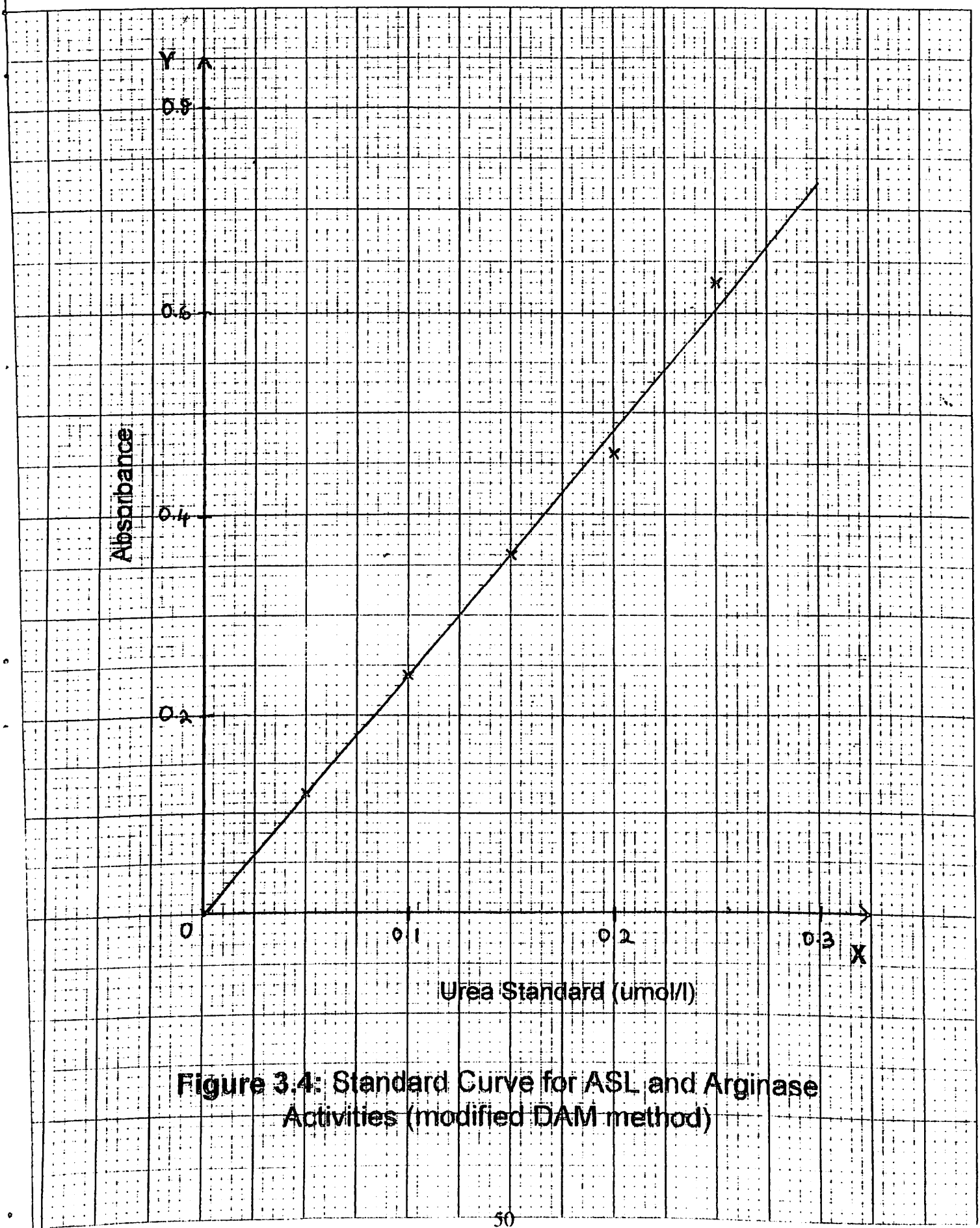

Figure 3.4: Standard Curve for ASL and Arginase Activities (modified DAM method)

Arginase activity:

Arginase activity was assayed according to the method of Herzfeld & Raper (1976) as described by Swamy *et al* (1983). The first step is to activate the enzyme in the sample. This is done by incubating 300ul of the 10% homogenates with equal volume of imidazole buffer, containing 56mM imidazole and 56mM $MnCl_2$ at ph 7.4, for 10 minutes at 50^0C. The activated preparations were then centrifuged at 3000rpm for 5 minutes, and the supernatants were used for measurement of enzyme activity. Enzyme assay was carried out in a total of 0.8ml of incubation mixture consisting of 100umol of L-arginine and 60umol of glycine buffer (both adjusted to pH 9.5 with 1.0N NaOH), and 0.2ml of supernatant after activation. After incubation at 37^0C for 10 minutes, the reaction was stopped with the addition of 0.2ml 50% trichloroacetic acid. For the controls, the trichloroacetic acid was added first together with the incubation mixture. The mixture was then centrifuged again at 3000rpm for 5 minutes and 0.5ml of supernatant was taken for estimation of urea. Urea was estimated using the DAM method as described for ASL activity above and the activity was expressed as μmol of urea formed per gram wet weight of tissue per hour (μmol urea/g wet tissue/hr).

Amino acid analysis:

Analysis of amino acid was done using the Biochrom 20 Plus Amino Acid Analyzer, which uses a PC-controlled liquid ion exchange system. This analyzer separates amino acids on a high-pressure ·temperature-controlled liquid exchange column which was continuously analyzed by ninhydrin photometric detection. Chromatographic control, detection and safety systems were performed via PC with fully integrated software. Amino acids were continuously analyzed by a fast and precisely controlled in-line reactor after mixing with ninhydrin, which was the most specific reagent available for flow reactions.

Ten percent (10%) tissue homogenate was used for this analysis and the sample was deproteinised first before analysis. This is accomplished using the compound 5-sulphosalicylic acid (SSA) which needs to be the highest purity to avoid contamination of the resin. For this step, 200ul of sample was mixed with 200ul of SSA. Then, 100ul of internal standard was mixed in the treated sample making it diluted 2.5 times. From this diluted sample, 20ul was injected into the analyzer column and the chromatogram was generated automatically. The concentration of amino acids was then calculated using the computer software based on the internal standard concentration. The printed-out results in the unit of μmol/l have to be multiplied by dilution factor (2.5) and finally calculated as μmol/gram wet tissue. Figure 4 shows a representative of amino acids chromatogram.

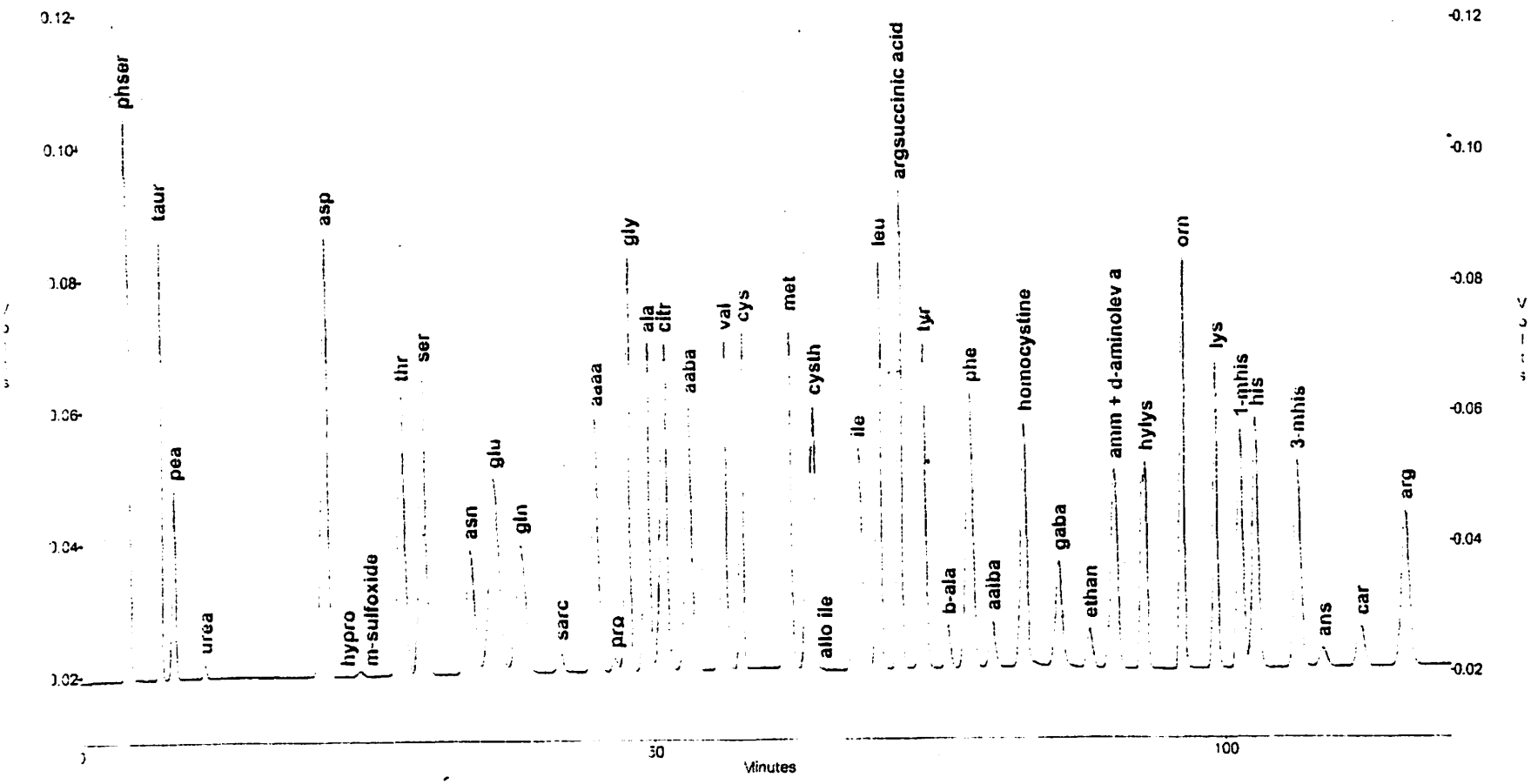

Figure 4: Example of Amino Acids Chromatogram

Statistical analysis:

Results were reported as mean ± standard deviation (SD) from 6 animals for each parameter calculated. Statistical analysis of results was done using independent t-test, using the SPSS software (version 11.5). p value of < 0.05 was taken as statistically significant at 95% confidence interval.

Results:

Results at a glance:

1) The concentration of ammonia increased significantly in all the three brain regions in rats subjected to hyperammonemic model.
2) NO concentration and NOS activity were significantly increased in all the regions of rat brain studied.
3) ASS activity was significantly increased in all the three brain regions in rats subjected to ammonia toxicity while ASL activity increased significantly in cerebral cortex and cerebellum.
4) Arginase activity was not significant in all the regions of rat brain studied.
5) Arginine, ornithine and glutamine concentrations increased significantly in all the three regions in rats subjected to ammonia intoxication whereas citrulline levels increased significantly only in cerebellum and glutamate concentration increased significantly only in brain stem. GABA levels were unaltered in all the three brain regions in rats subjected to hyperammonemia.

Ammonia concentration: The concentration of ammonia was significantly increased in all three regions of rat brain subjected to ammonia toxicity suggesting that the animals in the test group were having high ammonia level in the brain (Table 1). It can be clearly seen that there was a uniform increase in ammonia concentration in all the three brain regions in the experimental group (Figure 5.1).

Table 1: Ammonia Concentration in Different Regions of Rat Brain [a]

BRAIN REGIONS	CONTROL	TEST	% CHANGE [b]	p VALUE
Cerebral Cortex	3.37 ± 0.70	5.49 ± 1.29	+ 62.91	$p < 0.005$
Cerebellum	3.05 ± 0.44	4.54 ± 0.66	+ 48.85	$p < 0.001$
Brain Stem	2.44 ± 0.39	4.05 ± 0.65	+ 65.98	$p < 0.001$

[a] concentration is expressed as µmol per gram wet weight of tissue
[b] + = increase, - = decrease
results are mean ± S.D., n=6

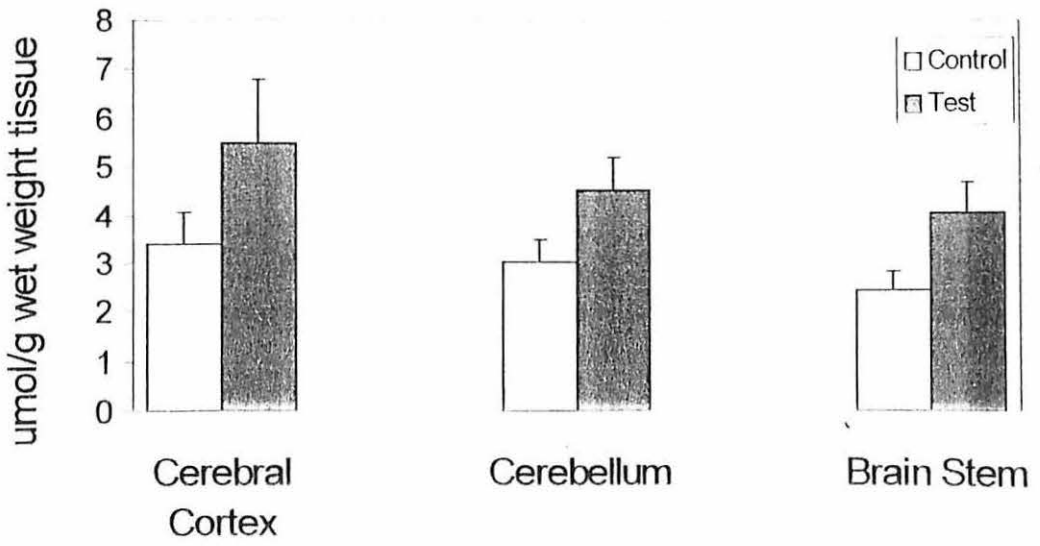

Figure 5.1: Ammonia Concentration in Different Regions of Rat Brain

Nitric oxide concentration:

The concentration of nitrate/nitrite (NOx) increased significantly in all three brain regions in ammonia toxicity group indicating that there was an increase in the nitric oxide concentration (Table 2). The increase of NOx in cerebellum was the highest when compared to the rise in cerebral cortex and brain stem (Figure 5.2).

Table 2: Nitric Oxide Concentration in Different Regions of Rat Brain[c]

BRAIN REGIONS	CONTROL	TEST	% CHANGE [b]	p VALUE
Cerebral Cortex	614 ± 74	832 ± 58	+ 35.50	$p < 0.001$
Cerebellum	761 ± 53	1153 ± 111	+ 51.51	$p < 0.001$
Brain Stem	640 ± 67	909 ± 90	+ 42.03	$p < 0.001$

[c] concentration is expressed as nmol per gram wet weight of tissue
[b] + = increase, - = decrease
Values are mean ± S.D., n=6

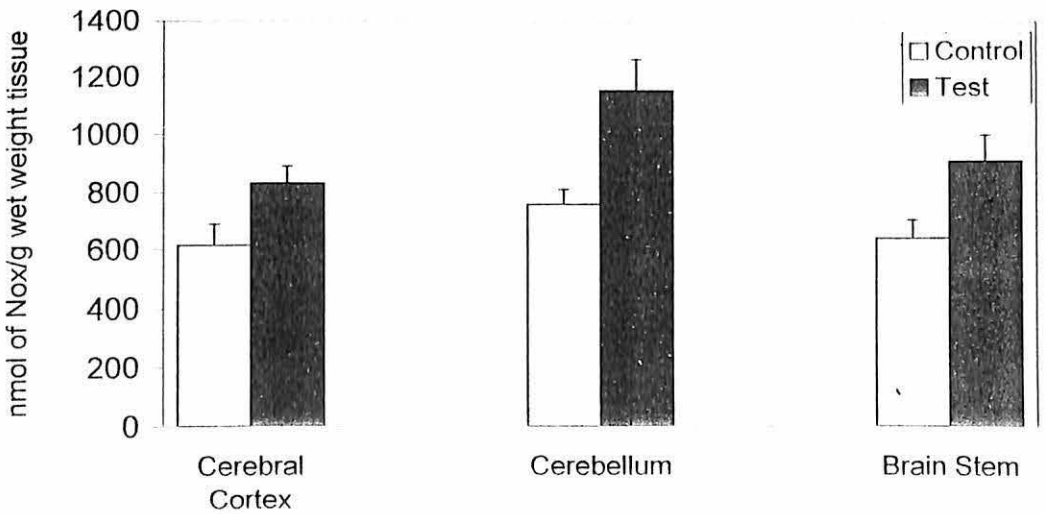

Figure 5.2: Nitric Oxide Concentration in Different Regions of Rat Brain

Enzyme activities:

The activities of the citrulline-NO cycle enzymes were noted to be higher in the rat brain subjected to ammonia toxicity (Table 4.1, Table 4.2 and Table 4.3). The activities of NOS and ASS were significantly increased in all three brain regions after ammonia administration whereas activity of ASL was only significant in the cerebral cortex and cerebellum but not in brain stem. The increase in NOS activity was highest in the cerebellum compared with the other two regions (Figure 6).

There was very minimal change in the activity of arginase in all regions of rat brain subjected to ammonia toxicity, which was statistically not significant, compared to the control animals (Table 5 and Figure 7).

Table 4.1: NOS Activity in Different Regions of Rat Brain [d]

BRAIN REGIONS	CONTROL	TEST	% CHANGE [b]	p VALUE
Cerebral Cortex	119.17 ± 11.29	161.33 ± 15.53	+ 35.38	p < 0.001
Cerebellum	159.00 ± 23.43	216.50 ± 28.59	+ 36.16	p < 0.003
Brain Stem	150.33 ± 16.46	209.50 ± 21.27	+ 39.36	p < 0.001

Table 4.2: ASS Activity in Different Regions of Rat Brain [e]

BRAIN REGIONS	CONTROL	TEST	% CHANGE [b]	p VALUE
Cerebral Cortex	1.44 ± 0.17	1.94 ± 0.26	+ 34.72	p < 0.001
Cerebellum	1.82 ± 0.19	2.40 ± 0.17	+ 31.87	p < 0.001
Brain Stem	1.15 ± 0.13	1.48 ± 0.25	+ 28.70	p < 0.02

Table 4.3: ASL Activity in Different Regions of Rat Brain [e]

BRAIN REGIONS	CONTROL	TEST	% CHANGE [b]	p VALUE
Cerebral Cortex	2.62 ± 0.20	3.11 ± 0.25	+ 18.70	p < 0.004
Cerebellum	2.34 ± 0.14	2.96 ± 0.23	+ 26.49	p < 0.001
Brain Stem	2.67 ± 0.25	2.83 ± 0.22	+ 5.99	NS

[d] activity expressed as nmol of NOx (nitrate/nitrite) formed per gram wet weight of tissue per hour

[e] activity expressed as umol of urea formed per gram wet weight of tissue per hour

[b] + = increase, - = decrease

values are mean ± S.D., n=6

NS = statistically not significant

Figure 6: Citrulline-NO Cycle Enzymes Activities in Different Regions of Rat Brain

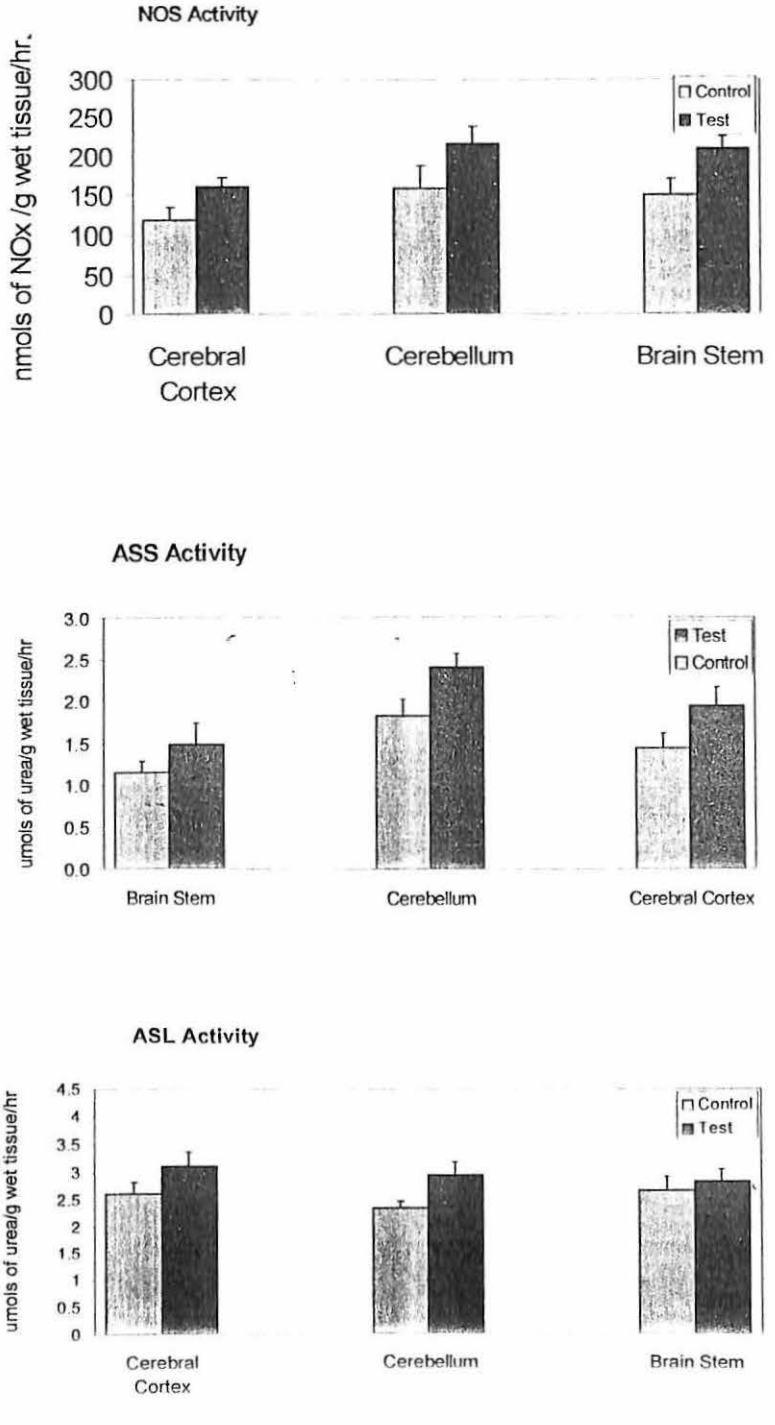

Table 5: Arginase Activity in Different Regions of Rat Brain [e]

BRAIN REGIONS	CONTROL	TEST	% CHANGE [b]	p VALUE
Cerebral Cortex	23.73 ± 3.95	23.92 ± 5.32	+ 0.80	NS
Cerebellum	32.35 ± 6.41	33.85 ± 3.62	+ 4.64	NS
Brain Stem	19.10 ± 4.65	21.72 ± 3.32	+ 13.72	NS

[e] activity expressed as umol of urea formed per gram wet weight of tissue per hour
[b] + = increase, - = decrease
values are mean ± S.D, n=6
NS = statistically not significant

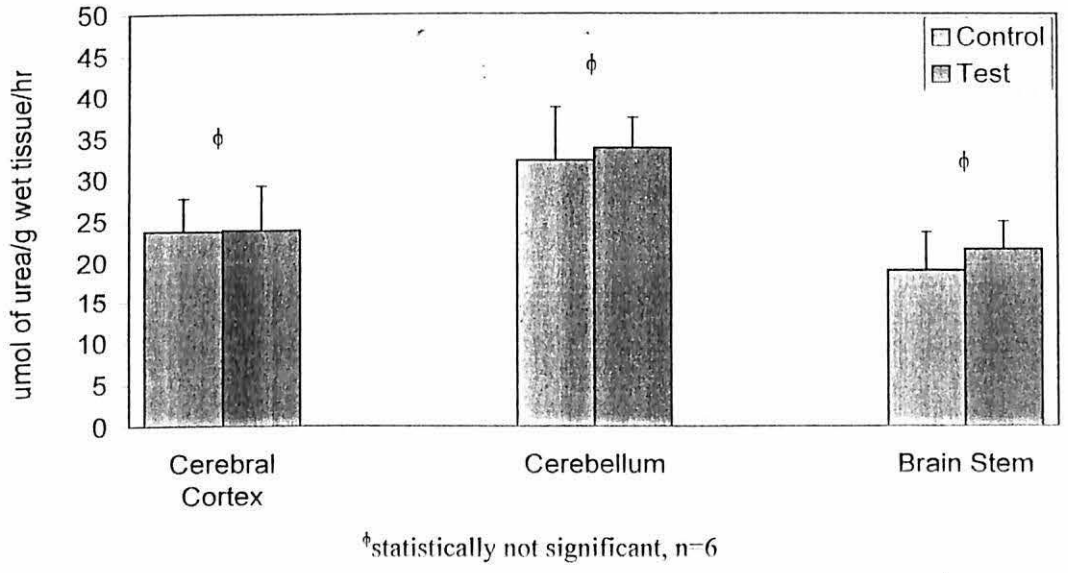

Figure 7: Arginase Activity in Different Regions of Rat Brain

Amino acids concentrations:

Tables 6.1 to 6.6 show the concentrations of amino acids in different regions of rat brain in both the control and test groups. Concentrations of arginine and ornithine were significantly increased in all three brain regions after administration of ammonia. However, the concentration of citrulline was only significant in cerebellum (Figure 8.1). Glutamine concentration was increased significantly in all three regions of rat brain in ammonia toxicity group. However, the concentration of glutamate was noted to be significantly increased only in the brain stem. GABA concentration, on the other hand, was not significant in all three regions of the brain but it was observed that there were reduced levels in cerebral cortex and cerebellum (Figure 8.2).